

Organization of the Methylamine Utilization (*mau*) Genes in *Methylophilus methylotrophus* W3A1-NS

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The organization of genes involved in utilization of methylamine (*mau* genes) was studied in *Methylophilus methylotrophus* W3A1. The strain used was a nonmucoid variant termed NS (nonslimy). The original mucoid strain was shown to be identical to the NS strains on the basis of chromosomal digest and hybridization patterns. An 8-kb *Pst*I fragment of the chromosome from *M. methylotrophus* W3A1-NS encoding the *mau* genes was cloned and a 6,533-bp region was sequenced. Eight open reading frames were found inside the sequenced area. On the basis of a high level of sequence identity with the Mau polypeptides from *Methylobacterium extorquens* AM1, the eight open reading frames were identified as *mauFBEDAGLM*. The *mau* gene cluster from *M. methylotrophus* W3A1 is missing two genes, *mauC* (amicyanin) and *mauJ* (whose function is unknown), which have been found between *mauA* and *mauG* in all studied *mau* gene clusters. Mau polypeptides sequenced so far from five different bacteria show considerable identity. A *mauA* mutant of *M. methylotrophus* W3A1-NS that was constructed lost the ability to grow on all amines as sources of nitrogen but still retained the ability to grow on trimethylamine as a source of carbon. Thus, unlike *M. extorquens* AM1 and *Methylobacillus flagellatum* KT, *M. methylotrophus* W3A1-NS does not have an additional methylamine dehydrogenase system for amine oxidation. Using a promoter-probe vector, we identified a promoter upstream of *mauF* and used it to construct a potential expression vector, pAYC229.

Methylophilus methylotrophus W3A1 is a restricted facultative methylotrophic bacterium (15) which belongs to the β subgroup of the proteobacteria (13). In addition to C₁ compounds (methanol, monomethylamine, dimethylamine, and trimethylamine), it is able to grow slowly on glucose as a source of carbon. This bacterium oxidizes methylamine via the periplasmic enzyme methylamine dehydrogenase (MADH) (16). MADH from *M. methylotrophus* W3A1 as well as MADHs from other bacteria consist of two large and two small subunits (1, 16, 26), and it has tryptophan tryptophylquinone as the prosthetic group (20). This MADH is distinguished from MADHs of other bacteria in that it apparently does not use the small copper-containing protein amicyanin as an electron acceptor (1, 29) but uses a c-type cytochrome instead (3). No copper-containing electron transfer protein was found in a related strain, *M. methylotrophus* AS1, and it is assumed that this bacterium does not synthesize these proteins (2).

Genes responsible for the synthesis of MADH (*mau* genes) have been cloned so far from facultative methylotrophic bacteria of the α subgroup of proteobacteria, *Methylobacterium extorquens* AM1 (4), *Paracoccus denitrificans* (5, 27), and *Thiobacillus versutus* (14, 31). In these strains, amicyanin is an obligatory component of the electron transfer chain for MADH (4, 27). In all three cases, the gene for amicyanin (*mauC*) was located immediately downstream of the gene for the MADH small subunit (*mauA*), with a hairpin structure between them. Eleven open reading frames have been found to comprise the *mau* gene cluster in *M. extorquens* AM1 in the order *mauFBEDACJGLMN* (4). The first eight genes (*mauF*

BEDACJ) have been found in the *mau* gene cluster from *P. denitrificans*, and they are highly similar to the corresponding genes from *M. extorquens* AM1 (17). The *mauBEDACJ* genes from *T. versutus* have been sequenced entirely or partially (14, 31), and they are also highly similar to their counterparts in the *mau* gene clusters from the two other facultative methylotrophs (14, 31). Properties and possible functions of Mau polypeptides are described elsewhere (4, 17).

In this report, we describe the cloning and sequencing of the *mau* gene cluster from *M. methylotrophus* W3A1.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this work are described in Table 1. All *Escherichia coli* strains were grown in Luria-Bertani medium in the presence of appropriate antibiotics as described by Maniatis et al. (18) except that the concentration of chloramphenicol for pAYC63 derivatives was 0.01 mg/ml. Isopropyl- β -D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) were added at 0.04 mg/ml. The *M. methylotrophus* W3A1 strains were grown in minimal Owens-Keddie (OK) medium (23) or the ammonia mineral medium described previously (10). The nitrogen-free medium used was OK medium in which sodium sulfate (0.2 g/liter) substitutes for ammonium sulfate. The concentrations of tetracycline, streptomycin, rifamycin, ampicillin, and kanamycin for growing *M. methylotrophus* strains were 0.01, 0.1, 0.05, 1, and 0.15 mg/ml, respectively, except when indicated differently. Methanol (1% [vol/vol]) and glucose (0.5%) were used as sources of carbon. Methylamine hydrochloride (0.5% [wt/vol]), dimethylamine hydrochloride (0.5% [wt/vol]), and

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
<i>E. coli</i> DH5 α	F ⁻ ϕ 80 (<i>lacZ</i>)M15 <i>endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1?</i>	New England Biolabs
<i>M. methylotrophus</i> W3A1		
NCIMB 11348 (W3A1-S)	Wild type, <i>slm</i> ⁺ <i>mox</i> ⁺	NCIMB ^a
W3A1-NS	Wild type, <i>slm</i> <i>mox</i>	21
W3A1-NS1	<i>slm</i> <i>mox</i> <i>rif</i> ⁻¹	This study
W3A1-NS2	<i>slm</i> <i>mox</i> <i>mauA1::Km</i> ^r <i>rif</i> ⁻¹	This study
Plasmids		
pRK310	Tc ^r <i>lacZ'</i> <i>mob</i> ⁺ IncP	8
pRK2013	Km ^r <i>tra</i> ⁺ IncColE1	8
pUC19	Ap ^r <i>lacZ'</i>	New England Biolabs
pAYC37	Ap ^r Sm ^s <i>mob</i> ⁺ IncQ	30
pAYC61	Ap ^r Tc ^r <i>mob</i> ⁺ IncColE1	4
pAYC63	Cm ^r <i>lacZ'</i>	5
pAYC152.1	Ap ^r <i>mauA</i>	6
pAYC208	Tc ^r <i>mob</i> ⁺ <i>mauFBEDAGLMN</i> (?) IncP	This study
pAYC229	Ap ^r P _{<i>mau</i>} <i>aphC</i> (Sm ^r) <i>mob</i> ⁺ IncQ	This study
pAYC233	Ap ^r P _{<i>mau</i>} <i>mauA aphC</i> (Sm ^r) <i>mob</i> ⁺ IncQ	This study

^a NCIMB, National Collection of Industrial and Marine Bacteria.

trimethylamine hydrochloride (0.5% [wt/vol]) were used as sources of nitrogen and/or as sources of carbon for growing *M. methylotrophus* W3A1 strains. These amines were purchased from Aldrich (Milwaukee, Wis.).

The rifamycin-resistant nonslimy (NS) strain *M. methylotrophus* W3A1-NS was selected as a spontaneous mutant on OK medium supplemented with 0.05 mg of rifamycin per ml.

Levels of streptomycin resistance were determined by streaking strains onto plates containing defined concentrations of streptomycin (0.005, 0.01, 0.02, 0.04, 0.1, 0.2, 0.5, 1, 2.5, 5, and 10 mg/ml). The MIC is defined as the streptomycin concentration in which growth did not occur (maximum time tested was 21 days).

DNA-DNA hybridizations. DNA-DNA hybridizations were carried out in dried agarose gels in accordance with the procedure described by Meinkoth and Wahl (22). The temperatures of hybridizations (6 \times SSC [1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0], 0.1% sodium dodecyl sulfate [SDS]) and washes (0.5 \times SSC, 0.1% SDS) were 68°C for homologous hybridization and 54°C for heterologous hybridization experiments with probes labeled via random primer reaction and 42°C when the oligonucleotide CA2 (GARGAY GAYGCSATGACSTAYCAYTG [6]) was used as a probe, labeled via the polynucleotide kinase reaction.

DNA manipulations. Plasmid isolation, *E. coli* strain transformation, preparative isolation of the DNA fragments from agarose gels, restriction endonuclease digestion, ligation, blunting of ends with Klenow fragment or T4 DNA polymerase, and the polynucleotide kinase labeling reaction were carried out as described by Maniatis et al. (18). Random primer labeling of DNA fragments was conducted as suggested by the manufacturer (Boehringer Mannheim Corp., Indianapolis, Ind.). Chromosomal DNAs of the *M. methylotrophus* W3A1 strains were isolated in accordance with the procedure of Marmur (19).

DNA sequencing. DNA sequencing was performed by the

dideoxy-chain termination method on both strands in the UCLA DNA Sequenator Core Facility on an Applied Biosystems sequenator. Plasmid pUC19 and pAYC63 (Cm^r) (5) were used as vectors for subcloning and sequencing.

Matings. Triparental matings were conducted as described previously (10). Plasmid pRK2013 was used as a helper plasmid.

Construction of a *mauA* mutant strain. *mauA* mutants were constructed by homologous recombination as described previously (25). The Km^r cassette from pUC4K was used as a selective inactivating marker. Plasmid pAYC61 was used as a suicide vector (4).

Computer analysis of DNA sequences. Computer analysis was carried out by using the PCGENE program (Genofit SA, Geneva, Switzerland). Algorithms used in this program package were those of Tinoco et al. (28) for hairpin structure searches and of Rao and Argos (24) and Eisenberg et al. (9) for the searches of membrane-associated or membrane-spanning helices.

Nucleotide sequence accession number. The GenBank accession number for the *mau* gene cluster from *M. methylotrophus* W3A1 is L26407.

RESULTS

Comparison of S and NS strains of *M. methylotrophus* W3A1. The original strain of *M. methylotrophus*, W3A1 NCIMB 11348 (formerly bacterium W3A1), produces large, mucoid colonies when it grows on methanol or trimethylamine. This has been referred to as the slimy (S) strain (21). On methylamine and glucose, it shows considerable colony heterogeneity, with colonies varying in size and mucoidity. We found that large colonies continued to produce large and small colonies after repeated transfers onto fresh glucose or methylamine medium, whereas small colonies inherited their morphology when methylamine or glucose was used as the growth substrate. Both large and small colonies produced large mucoid colonies on methanol and trimethylamine. The second strain of *M. methylotrophus* W3A1, the so-called NS strain, was selected earlier by McIntire and Weyler (21). They have shown that the NS strain retains all growth characteristics of the S strain such as maximum growth rate and maximum yield on methanol, methylamine, and trimethylamine (21). However, the NS strain has been propagated since 1987 in medium containing methylamine or trimethylamine, and recent analysis has shown that it has lost the ability to grow on methanol. In addition, the NS strain is not able to grow on the plates with the ammonium mineral salts medium, whereas the S strain can.

To determine whether the S and NS strains are identical, chromosomal DNA was isolated from the NS strain and different lines of the S strain which differ in colony morphology on OK medium supplemented with methylamine or glucose. These DNA preparations were digested with *Hind*III and separated on an agarose gel (Fig. 1A). The digest patterns show an identity of the NS and S strains. Since an initial objective of this work was to clone the *mau* genes from *M. methylotrophus* W3A1, the gel shown in Fig. 1 was hybridized with the oligonucleotide CA2, which has been successfully used for cloning the *mau* genes from *P. denitrificans* and *M. extorquens* AM1. Hybridization experiments with specific probes detected two bands with the same size in all cases (Fig. 1B). We chose to use the NS strain, since it gives colonies of a single morphological type, which is advantageous for genetic analysis.

Cloning and nucleotide sequence of the *mau* gene cluster from *M. methylotrophus* W3A1-NS. Two partial clone libraries were constructed in the vector pRK310, using the fractions of

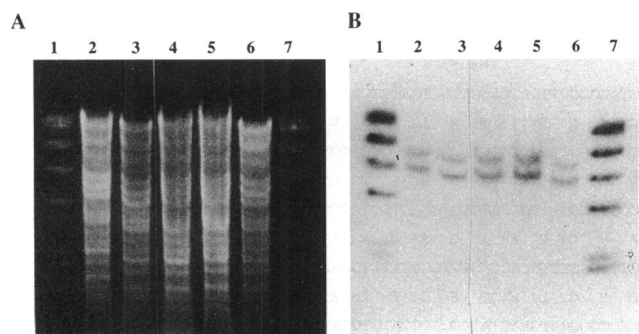


FIG. 1. (A) *Hind*III digests of the chromosomal DNA preparations from the initial strain W3A1-S (lane 2), a large colony selected on methylamine (lane 3), a small colony selected on methylamine (lane 4), strain W3A1-NS (lane 5), and strain W3A1-NS1 (lane 6). Lanes 1 and 7, *Hind*III digest of phage λ DNA. Sizes of fragments from the top are 23, 9.4, 6.5, 4.3, 2.3, and 2.0 kb. (B) Hybridization of the same gel with the labeled CA2 oligonucleotide.

the *Hind*III digest of the NS strain chromosome which hybridized to the CA2 oligonucleotide. These two fractions had approximate size ranges of 6.4 to 6.6 and 7.0 to 9.0 kb, and they were collected in a well in agarose gels as described by Maniatis et al. (18). Clones with positive hybridization to this probe were identified in each library. Plasmids were isolated from these clones and subcloned, and areas where the oligonucleotide had bound were sequenced. In both cases, the oligonucleotide bound to DNA of similar sequence but not to a putative gene for the MADH small subunit. Therefore, a 0.7-kb *Pst*I fragment containing the entire *mauA* gene from *M. extorquens* AM1 (from pAYC152.1 [6]) was used as a probe against chromosomal digest of the NS strain. It hybridized to a 8-kb *Pst*I fragment. A fraction of NS strain chromosome *Pst*I digest with fragments of about 8 kb were isolated from an agarose gel, and these fragments were cloned into the pRK310 vector. Positive clones (approximately 1 per 450 white colonies) were identified via hybridization with the labeled 0.7-kb *Pst*I fragment. The only orientation of the 8-kb *Pst*I fragment insert obtained was that in which the *mau* genes cannot be transcribed from the *lac* promoter (plasmid pAYC208). All attempts to obtain transcription of the *mau* genes under control of the *lac* promoter in pRK310 or pUC19 by recloning were unsuccessful. The restriction map of the 8-kb *Pst*I fragment is shown in Fig. 2.

We sequenced 6,533 bases of the 8-kb *Pst*I fragment (Fig. 3).

Eight open reading frames were identified in the sequenced area. The first 315 nucleotides upstream of the first open reading frame do not encode any polypeptide in either strand.

Computer analysis of the *mau* gene cluster organization and polypeptides encoded by the *mau* gene cluster. The eight open reading frames identified by sequencing were determined to be *mauFBEDAGLM*, based on identity with the corresponding genes of *M. extorquens* AM1 (4). The major difference between the *mau* cluster from *M. methylotrophus* W3A1 and those from facultative methylotrophic bacteria is that *mauC* (the amicyanin gene) and *mauJ* (a nonessential gene with an unknown function) are missing from the former. MauF (285 amino acids [aa], 30,429 Da) and MauE (190 aa, 20,159 Da) are predicted to be membrane polypeptides, each with four predicted transmembrane helices. The remaining polypeptides are predicted to be periplasmic. The MauB (405 aa, 45,243 Da) polypeptide is the MADH large subunit. The MauA (187 aa, 20,237 Da) polypeptide is the small subunit polypeptide, and it has the unusual leader sequence structure found in other MauA polypeptides (17). It is 58 aa long and contains a high net positive charge near the signal peptidase recognition site. The MauD (211 aa, 23,083 kDa) polypeptide appears to have a leader sequence without positively charged amino acids. MauGs from both *M. extorquens* AM1 and *M. methylotrophus* W3A1 are predicted to contain two putative cytochrome *c* binding sites, and MauMs from both these species are predicted to contain four [4Fe-4S] clusters (indicated in Fig. 3). MauG and MauM from *M. methylotrophus* W3A1 have molecular masses of 37,265 Da (335 aa) and 23,868 Da (223 aa), respectively. The MauL polypeptide (174 aa, 19,085 kDa) from *M. methylotrophus* W3A1 has a lipoprotein signal peptidase recognition site. A potential initiator Met was found downstream of *mauM*, and it could be the beginning of *mauN*, which is located downstream of *mauM* in *M. extorquens* AM1 (4), although the extent of sequence is not sufficient to identify this gene.

Several hairpin structures were identified in the *mau* gene cluster from *M. methylotrophus* W3A1, inside genes as well as in intergenic spaces. Hairpin structures with energies of production more than 10 kcal (ca. 42 kJ) mol are indicated in Fig. 2 and 3.

Construction of the *mauA* mutant and its properties. A *mauA* mutant was constructed by using homologous recombination (25). The *Km^r* cassette from pUC4K was inserted into *mauA* in such an orientation that *aph* and *mauA* were transcribed in the same direction, and this construction was introduced into the chromosome of *M. methylotrophus*

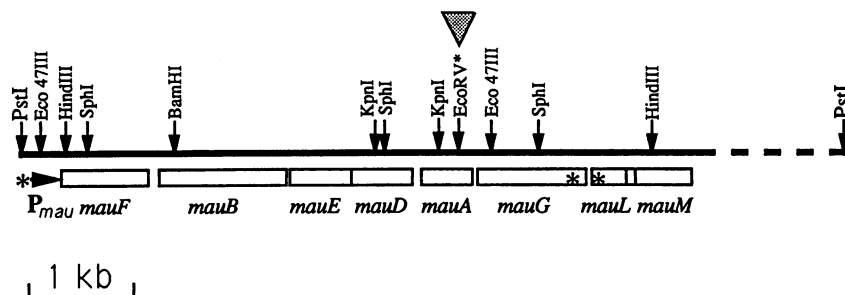


FIG. 2. Physical and genetic map of the 8-kb *Pst*I fragment containing the *mau* operon of *M. methylotrophus* W3A1. The sequenced part of this fragment is shown as a solid line; the nonsequenced part is shown as a dashed line. Restriction sites with asterisks are not unique on this fragment. *, sites of hairpin structures. The triangle at the top indicates the site of the kanamycin resistance gene insertion in the *mauA* mutant. *P_{mau}* and the arrow indicate a promoter of *mauF* and the direction of transcription from the promoter.

1 CTGCAGATGGCTATAAAACAGCCAAAAAAGTTCTTCGGCTATATCCGCCGTAATATGTAAGTTCGGGTTTTCCTCGCTGCTAGCTGGCTCGCCGGCCAGCTGCTCTCGGTTTATAGC
121 CCCTTCAGATATCTCCGGCATTTCTTCAAAAGAGGGGCTTTTTCATAGTAAAAATGGCATGACTTGGTCTGCATGGCGTGTCTTAAAAAGGATATGCCGATTCTTGCTAAA
241 GCGCTGGAATCAAGGCCGCTATATTCATGTTTTCATTCAAAACAAAGCTAAGCCAGCAGGCCGAAGGAGGAAATATGAGTATGAAATAGGGGCCACTGCCATGAGCCGTTCTGTCCTCAAG
M S M N I G A T A M S R S S Q
MauF
361 CTTACGCAGTCGAGACCTGTGTTCTCTGATGCGTATCATTTTTCAAAAGCACAAATCGACTGGTACGCGCTTCATCATGATGCTGACCGCAGTTGCCAGCGGTGTGTTTGTGCGCGCGTCA
A Y A V E T C V P D A Y H F S K A Q S T G T R F I M M L T A V A S G V F A G R V
481 TGCAATAGCAGATGTCAGTCGAGATGGCGCTCACTGGTTTGTGTGTTGGTGTGCTTTCGTTGGGTGGTTTGTCTGTCTACCTGGTGGCTTGTGGTTACTCAAGTTTAAAGTTTATGCGAC
M H S T M S V E M A L T G L F V V L A F V G G L L S T W S P C G Y S S L S L L R
601 CGGCTGGACGTTATTCATTAGCGCTGTTCACCGTTGGCGCGCTACCTTCTTCACGCATGCGCTGGGGTATGCCATCGGTGCCGTTGTACTCGGTGGTGGCTCGGTGGTATCAGCTGGC
P A G R Y S L G A V T R W A P T F F T H A V G Y A I G A V V L G G A L G G I S W
721 TGCTGTTTGGCGAGTCGCATGTCAGTATGCGGTGATGGCTGGCGACCTGGCGATGGTTGCTACGGTTTGGCACCAGTTTGGCTTTTGAATAATGCCCTTATCCGCAACCGCGTGGCAAG
L L F A D V P L Q Y A V I G L A T L A I I G Y G L H Q F G F L K M P Y P Q R R A Q
841 TGCCACATGACGCACGATTCGCTTCCGTTTCATCCGTGATGGCTGTGTATGGCTTTTTCATGGCGTGAACCTATCTACCTATGTGCAAACTCCGATGCTGTACATCGTCACTGGTG
V P H D A R F R F R S S V I G L L Y G F S L G M N Y L T Y V Q T P M L Y I V T G
961 TGGCTTGTGAGTGGTGGCGTCAAGGCTGGCATGGCGTATCGCGTATTCATATCGGTGCTGCTTGGCAGTGGCGGTAAATTTCTTACCTGTTAAGGATCAGTCAGTTCAAGCCT
V A L L S G G V K A G I A V I A V F N I G R C L P V A V N F L P V K D Q S V Q A
1081 GGTGGCCGCTTGGCAGGAAATGTCAGTTGAAGTTGATGGTTTCTGTTATGGCGATCGCTTACGACGCTTTAATGCTGGTGTGTTGTAAGTCAGTAGCAACACCAGACGCAATAGCA
W L A R W Q E S A V E V D G F L L L A I A S A A L M L V M L *
1201 AGTTCTATCCATTAAAAATATTAGGAGGATTTATGACAACATTCGATCATCCATCGATGATTCGTCAACCAAGCCGACAGGTTTGGCGGGGGGGTGGTTTGGCCGCACTCATGTTA
M T T F D H P S M I R Q P K P T G L A G G L V L A A L M L
MauB
1321 TCCAGTAGCCTGGCCTTGGCAGATGCAACGCCTAACCAGCTTGGTTTCAGAAATACGGCCAAAGCTACAGGACGAGACGTCATCGCGATGACCCGCCAGTACTCCAAGCGCTCTAT
S S S L A L A D A T P N Q L G S E I T A K L Q D E T S I A I A P A S D S K R V Y
1441 GTGCTGGATCCAGGTAATTCATATGACCTCTACCGTCTATACGATAGATGGCAATCAAGCAAAATTAATGGGCATGACCGATGCGCGTAAATTGGCTAACGTCATGGTAGCGTCAGAT
V L D P G N F H M T S T V Y T I D G K S S K L L G M T D A G K L P N V M V A S D
1561 GGCAAGTTTGTGGCATCGCAACACCAGTACTCCCGCTGGCCCGTGGCAACGTCATGACTACCTGGAGTTGATCGACACCAAAACGTCATCAACCCATTGGCGATATCGATATTCCT
G K F L A I A N T M Y S R V A R G K R D D Y L E L I D T K T H Q P I A D I D I P
1681 GAAGTGGTTTGTGACCGGTGTGTTGAGCGTACGGCGGGTGTGAGTGGATGACAAGCACTGTGTGTTCCAGCAATTCACCGTACACAGCGGTAGGCTTGGTCGATCTGCAACAA
E G R F L T G V F E R T A G L S V D D K H L L F Q Q F S P S P G V G L V D L Q Q
1801 AAGGCTTTCGTGAAATCATGAACGTCGCTGATTTGTTACCATATTTTCCCAACGGCTAACCAGAACTTCTTCATGCACTGTAGAGACGGTCTTTGATGAGTTACCTATGACAGCAAG
K A F V K I M N V P D C Y H I F P T A N Q N F F M H C R D G S L M Q F T Y D S K
1921 GGCAACACCAAGCAGAACCCCAAGATATTCATGCGCAAAAAGAGTATCTGCTGAATAACCCCTTATTAATCAAACTCCAATAATCACTGACCTGGCCGACCTATGAAAGCAAGATT
G N T K Q K P T K I F H A E K E Y L L N N P Y S N S N N H L T W P T Y E G K I
2041 TTTACGGCGAAATATCTGAGAGCGCGCGGAGTTCTCAAGCCTATCGAAGTGTTCACCGCAAGGAGAAAGCCGACAAATGGCGTCCGGGTGGCTGGCAGACCATGGCTTCCACAAA
F Q A K L S E S G A E F L K P I E V F T D K E K A D K W R P G G W Q T I A F H K
2161 GCGCGTAACGAGCTCTATTGCTAGCAGACCAACGTAAGAAATGGACACACAAATGGCTAGCCGTTTGTGTTTGTGGTGGATGCGACCTTGGCAACGCTGCGCCGTATCGAGTTG
A R N E L Y L L A D Q R E K W T H K L P S R F V F V V D A T S G K R L R R I E L
2281 AAGCATGAAATCAACTCTATCGCGTACGACGAGACGACGCGTACTGTACGCGGTGTCTGAAGAGCCAAACACTGTTCACTTTTGTATGCTGTGAACGCGCAAGCCTTTGTCCAGT
K H E I N S I A V S Q D D K P Y L Y A V S E E A K T L F T F D A V N G K A L S S
2401 ATTGATGAACGGCGGTGCACCTTCCATGATTTTCATGCGGATAAGTGAAGACGCTATGGAATCGATGGTAAATGATCCTACGGTAGCGATGTTTGCCTCGCTGTTCTGCGCGCTGGT
I D E L G R A P S M I F I A D K * M E S M V N D P T V A M F A S L F V A L V
MauE
2521 CCTGGCCGCGCAGCGATTCCAAAGCTGAGAAGTCAGGATGAGTTCTTGGCGTGGTGGCCAAATTCACCAATGCTGCCAGGCTTCTGTTGGCACCGTTTGGCAAAATGCTGCCATGGCT
L A A A A I P K L R S Q D E F L G V V A N Y K L L P G F L V A P F A K L L P W L
2641 GGAATCGGGTGTGCCATGTCTTGTGTTGCCATCACTGCGTGTCTGGCCGCTGTGTGGTGGCGCGCTTTCATGCTGTCTCTTTCGCCATCGCGGTGAATGTTGGCCGCGGCGG
E L G C A I A L L V P S L R V L A A C V A A A L F M L F S F A I A V N V G R G R
2761 CACCCACATTTGATGTGGATGCGTAGCTGCGCCAAACAGCATGAGCGGTATCGGTATGTTTTCATGTGCTGAGAGCCCTGGCGCTGGCTGGCATGAGCTTGTATGTCGCTGCTGTGCGGTT
T H I D C G C V R R P T S M S R I G M F H V L R A L A L A G M S L Y V A A V P L
2881 GGAGATATCTGGCATTAGCATCGAATCAGCCCTGACTGCATGCGCTTGGCGGTGATGTTGCTACTGATTTACATGCGCGCAGACCTGATGGTGGGCTTTTCAGCGACCAAACTAAAT
E I S G I S I E S A L T A L A S A V M L S L I Y M A A D L M V G F P A T K H K L
3001 AGAAATCTATAAGCAATTCAAATGACTAGTGAATCTGTATGTCATCAAAATGTGCTGTGTGGGAGCATTTTTGGCATGCGCAGCGTTAATGCTGGCGGTGATCCGTCAAATGGTT
E I Y K G N S N D *
M T S G I L I A S N V L L W G A F L A L A A L M L G V I R Q I G
MauD
3121 TATTACATGAACGCTCAGCACCGCTGGCGCCATGATGATAGACACCGCCGGATTCGGCGAACGTTTCACCGATTTCATATGTGAACACCTTCGACCGCGAGCCGGTATTTGGTGGCC
L L H E R S A P L G A M M I D H G P D V G E R S P I F N V N T F D G E P V L V G
3241 GCTCCATACCCAGGCGCTCTAGCTTGTGATGTTTACCGCCCATCTGCGCGATCTGCCAGAACTGTTGCTTATCATCCGCTCAGTCGCAGCAATGAAGAGACCGACGATTC
R S I T P G R P S L L M F T G P S C P I C Q K L L P I I R S V A A I E E T D V I

FIG. 3. Nucleotide sequence of the 6,522-bp *Pst*I fragment of the *M. methylotrophus* W3A1 chromosome containing the *mau* operon. Putative Shine-Dalgarno sequences are double underlined, hairpin structures are underlined, and putative leader sequences are italicized. In boldface are shown the following in MauA, the two tryptophans participating in the synthesis of the tryptophan tryptophylquinone cofactor; in MauC, the putative heme *c* binding sites; and in MauM, the putative iron-sulfur cluster signatures.

3361 TGATCAGTGATGGTACCCAGGCCGAGCATCGCCAGTTCCTTAAAGATCACCCGCTGGATGGCGAGTGTGATGTGGTTTCTGCCGAGATCGGCATCGCCTACCCAGGTATCAAAGTACCTT
L I S D G T Q A E H R Q F L K D H P L D G E L Y V V S A E I G M R Y Q V S K V P

3481 ACGCGCTGCTGCTGGATCAAGACGGCAAAATCCTGGCAAAAGCCCTGTGCAATACCCGTGAGCATGTAGAAAGCCCTGTTGAGACCATTCGCGAAGGTCAITTAACCTTGCGAAGTACC
Y G V L L D Q D G K I L A K G L C N T R E H V E S L F E T I R E G H S T L Q N Y

3601 TCAAGGATGAAAACTGCACCTAAATTTAAACAAGTACCGCGAACAAGTGCATTAAACCCCGGTTACGTTATTGATTACGAGAGAGTAAACGAAATGAAAAAGATACTGGGTTTG
L K D E N T A P K F K Q V T A N K V H * M K K D T G F
MauA

3721 ACTCAAAATTGAGAAGTTGGCAGCACCACCGCCAGCAAAACCGGCCGCGTGGTTTATTCGGCCGCTTGGTGGCTTCTGGTGGTTCTGGCTTGTATACCCCTGTTGCCGGTAGACC
D S K I E K L A R T T A S K T G R R G F I G R L G G F L V G S A L L P L L P V D

3841 GCCGCTCACGCTTGGGTGGCAAGTGCAGGCGGCTACCCAGGTAACCTGACCAGAAGCGGCTTCAAGCCACAGGACAAAGACCCATAAGCCCTGTGAGTACTGGCGCCATTGTACGATTG
R R S R L G G E V Q A A T T G N L T R S G F K P Q D K D P K A C E Y W R H C T I

3961 ACGCAACCTGTGTGACTGCTGCGCGGTACCTTGACCTCTTGCCCGCCAGGACAGCGCTGTCTCAAGTTCATGGGTGGCCAGTTGTTTACAACCCAGGTAGCAGCATTCTGA
D G N L C D C C G G T L T S C P P G S S L S P S S W V A S C Y N P G D Q Q T Y L

4081 TTGCCTACCGTACTGCTGTGGCAACAGACTTGGCGCGGTGTAACTGCGTCAACACGCAAGGTGAGCTGCCAGTGTACCGTCCAGAGTTCAACAACGATATCGTCTGGTGTCTTGGCG
I A Y R D C C G K Q T C G R C N C V N T Q G E L P V Y R P E F N N D I V W C F G

4201 CTGACAATGATGCAATGACTTATCACTGCACCATTTACCTATCGTTGGTAAAGCTAGTTAAGGTAAAGCAAGCTAACGCTTGTGTTAAAGAGAAATCAGTATGTTGTTAGACATTTG
A D N D A M T Y H C T I S P I V G K A S * M L F R H L
MauG

4320 GTTCTAATCATAAGCAGCTGATGGTGGCAATACTGCCTGGTCCGCAAACTCTCTCCGAGGGAAAAGTTCAAGCGCCAGACAGTATTCGGCACCCCTGTCTAACCCCTGACCCCTT
V L I I S T L M V A N T A W S A N L P P R E K F K R P D S I P A P L S N P L T L

4441 GAAAGAAGCCACGCTGGGAAAAACCTGTTTTTCGACCCAGCGCCTTTCCAGGTGAGCGGGATGGCTTGTGCCACTTGTCACTCCCCAGATCAGCGCTGGAGTGATGGCGCCACGTTGCCA
E K A T L G K T L F F D Q R L S R S G M A C A T C H S P D Q R W S D G R T L P

4561 TTGCAGGCAGAAAGCGTAAGCAATGCCCGTCCGACGCCGACAGTATTAAACAGTGCCTGGTTAAGTGCACCTCATGTGGATGGCGCGCCACTACCTCGAAGAACAGCGCGTGTGGCG
L Q A E S V S N A R R T P T V L N S A W L S A L M W D G R A T T L E E Q A V L P

4681 ATTACCACGGCACAGAAATGAATTTTGACCTAGCCAGCCTGGTGAAGTGTATTACAGCGAATTGAGGGCTATCGGCCCTTGTTTACCCAGGCTTTTGGCGATGACAGTATTAGCCAGCAG
I T T A H E M N F D L A S L V S R L Q R I E G Y R P L F T Q A F G D D S I S Q Q

4801 CGGATCAGCAGGCGCTGCCAGTTTCCAGCGTACGCTGGTGTCCAAATATTGCCCGCTTTGACCGCTGGGTAGCCGCGGACGAAACAGGCCATCAGCGAATCCGCCAAGCGTGGTTTGTCT
R I T Q A L A S F Q R T L V S N I A P F D R W V A G D E Q A I S E S A K R G F A

4921 GTGTTTAAACGATAAAATAAAGCTAATTGTGTCGCTTGCCATAGCTCATGGCGGTTTACCAGCATAGCTTTTCAATGACATTGGCCTGCCGAGCAAAAGTCTAGGCCGAGGCCCAAGTG
V F N D K N K A N C V A C H S S W R F T D D S F H D I G L P S K D L G R G A K V

5041 CCTTCGCAAGTGACCTTGATGACGATGCGTTTAAACCCCTCGTTGCGTGACCTGTGATTTGACGGGCCCTATATGCATGATGGCTCCATCCGTGGCGTGAAGAACAGTGATCAAGCAT
P S Q V T L M Q H A F K T P S L R D L S I D G P Y M H D G S I R G L K T V I K H

5161 TACAAAAGTAGGCGCATCCAGCGGAGAGCCTTTCCAAGATATGCAAGATTGCAACTGTGCAATGCAAGAAAGTGACCTGATCGCTTTTATCCAAGCCCTGGACGGTGGCTTGCCT
Y K S E A I Q R E S L S K D M Q K F E L S N L E E S D L I A F I Q S L D G G A L

5281 AAAATTACGCAACCGATGATGCGCTGAATAGCAATCGTTGAGTGGTGGTGAAGCTAGCAAAAGTAGATGAAAACGTTTTTGGCTGGATTAAAGAGCGCTCGCGCAAACTGTCCGCTGGAT
K I Q A P M M P E * M K T F L P G L M S A S R K L S A W I
MauL

5401 AGGCCCTGTATGCCTTGCTGGTGTGCTGGCGCTGTACCTCGCCAGCTGTAGCGCGCGAGTCAGCGCGCGTGAACAAATCTGCCAGTGATCCTGCGCATAGTATCGCATGACACTGTC
G L Y A L L V L L A L Y L A S C S A A S H A P L K Q S A S D P A H S H R M T L S

5521 TGACCATGAGTTTGCGCCTGTGGATTTTGGAGCCGCAACAGGCGACACATTACCATCCGCAACCGTTCCGATATTTGCGACAGCATTTATGTGACTTACC CGGACGGCACCATTGGTGAA
D H E F A P V D F E P Q P G D T I T I R N R S D I S H S I Y V T Y P D G T M V N

5641 TCTAGGGGTACAAACGCGGGTACGACGGTGCATTGGCAGGTGCTTCCGATGCCAAAGCTGAGTTTGTGCTGCAATGCTGATACATCCGATTATTCGCGCCAAATTTGCTGGTAAATGC
L G V Q T P G T T V H W Q V P A D A K G E F V L Q C W I H P I I R A N L V N A

5761 TGCCAATTGTGATCTTACGCTTTTAAAGTCTGCTTGGCAAAAGTTCACGCGCTACGAGATTCTCGATGGTTCGACAAAACATCTGTAGCAGCAGAAATCGCCGAGCCTGAGCGCGGTAAAG
A N L S S S A F K S A L P K F T R Q E I L D G R Q N I C S S R N R R A * M V D K T S V A A E I A E P E R R K
MauH

5881 TTTTTCGCAAGGTGTTCCGCCAGTTAGGGCGGCCACTGTCAATTACCATGTTTGGTAAAGCGGTGCTGATTCGCGTCCGCGACGCGCGGCGAGCTACTCAGCGCCAGGTGCCTTGC
V F R K V F R Q L G A A T V I T M F G K A V L D S R P A R A A S V L R P P G A L

6001 CTGAGCCAGAGTTCAATGCCCGCTGTATCCGCTGTGGCTTGTGCTAGAAAGCTTGGCCCTATGATATTTTGACACCTGGCCAGCTGGAGTGATCCGCGCGGACCTGGTACGCCTTATTTTG
P E P E F N A A C I R C G L C V E A C P Y D I L H L A S W S D P A P T G T P Y F

6121 TTGGTCTGACGACCCCTTGCCGATGTGCTGATATCCGTCGCGACGTGCTGTCGACAGGGGCAATTGAGCCCATTTGCTGACTGATATTAAGAAAGCCGATATGGCGGTGGCGGTAT
V G R T D P C R M C P D I P C A R A C P T G A L S P L L T D I K K A D M G V A V

6241 TGGTAGGCATGAGACCTGCCTGAATTTAAAGGCTCACTTGCAGCATTTTGGTGGCGGTCTGCCCCATTATTGGTGAGGCGATTTCGCTCAAGCAGATTAAAAATGAACGCGCGGTGT
L V G H E T C L N Y K G L T C S I C V R V C P I I G E A I S L K Q I K N E R G V

6361 TGCAGATCCCTACCGTCGATAGCAGTAAATGTACCGTTTGGCGCACCTGTGAAAAACACTGCGTGTGTGCAAGCGCGGATCCGCGTGTGCCACGCGAGTTGGGTCTGGGTGTCGAAGGT
L Q I C P T V D S S K C T G C G T C E K H C V L S E A A I R V L P R E L G L G V I G

6481 GCACAATCGGTAGTGGTGGTAAAGCTATGCGAGCTGTCAAGCCATACT
A Q S V G R W * M R R L Q A I

FIG. 3—Continued.

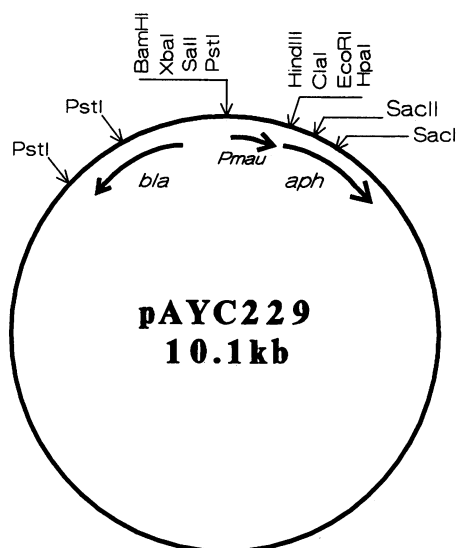


FIG. 4. Physical map of pAYC229. *bla*, ampicillin resistance gene; *aph*, streptomycin resistance gene; P_{mau} , a putative promoter from *M. methylotrophus* W3A1 transcribing *mauF*.

W3A1-NS in the suicide vector pAYC61. Two Km^r Tc^s Ap^s colonies were selected for further work. To prove that they have the correct insertion of the Km^r cassette the chromosomal DNA was purified from these strains and hybridized with the labeled Km^r cassette and the *Sph*I fragment carrying *mauA* which was used for construction of the mutant. In both cases, the *mauA* mutants showed hybridization of the same fragment, which is 1.4 kb larger than the fragment hybridizing with the *mauA* probe in the wild-type strain *M. methylotrophus* W3A1-NS (data not shown), confirming the correct insertion.

The growth spectra of the *mauA* mutant and the wild-type strain *M. methylotrophus* W3A1 were studied. As noted previously, both strains were unable to grow on methanol but retained their ability to grow on glucose. The *mauA* mutant in addition lost the ability to grow on methylamine and dimethylamine as sources of both carbon and nitrogen. The ability to utilize those compounds as nitrogen sources was tested on nitrogen-free OK medium with glucose as a carbon source. This mutant grew on trimethylamine as a source of carbon, but it could not utilize trimethylamine as a source of nitrogen. The growth rate of the *mauA* mutant on trimethylamine as a source of carbon was approximately one-third of that of the NS wild-type strain.

Identification of a promoter upstream of the *M. methylotrophus* W3A1 *mau* gene cluster. To determine whether the 315-bp region upstream of *mauF* contained a promoter, the 363-bp fragment between the *Pst*I and *Hind*III sites (Fig. 2)

was cloned into pUC19 and then recloned in the promoter probe vector pAYC37 (30) between the *Bam*HI and *Hind*III sites. The cloned fragment contained the part of the pUC19 polylinker from *Bam*HI and *Pst*I. The resulting plasmid, called pAYC229, is depicted in Fig. 4. It has the *aphC* gene transcribed from a putative *mau* promoter (P_{mau}). *E. coli* containing pAYC229 did not show resistance to streptomycin, suggesting that P_{mau} does not function in *E. coli*. However, P_{mau} seems to be a strong promoter in *M. methylotrophus* W3A1. Cells containing pAYC229 grew normally on plates containing up to 10 mg of streptomycin per ml, whereas cells containing the pAYC37 promoter-probe vector would not grow on plates containing more than 0.5 mg of streptomycin per ml for the *M. methylotrophus* W3A1-NS host.

A 1,081-bp *Sph*I-*Eco*47III subfragment of the 8-kb fragment carrying the *mauA* gene was cloned into pAYC229. The resulting plasmid (pAYC233) was introduced by a triparental mating into the *mauA* mutant strain. The analysis of 100 randomly picked transconjugants showed that all of them regained the ability to grow on methylamine.

DISCUSSION

MADH from *M. methylotrophus* W3A1 has been shown to use cytochrome *c*₅₅₂ instead of amicyanin as an electron acceptor (3). Therefore, it is interesting to compare the organization of the *mau* cluster from this bacterium with *mau* clusters from bacteria which contain MADH that couples to amicyanin. The *mau* gene cluster from *M. methylotrophus* W3A1 was cloned and sequenced, and although in most respects the gene organization is similar to that in the other known *mau* clusters, two genes are missing. These are *mauC*, the gene for amicyanin, and *mauJ*, which has an unknown function. In the facultative methylotrophs, *mauCJ* are found between *mauA* and *mauG* (17).

The *mau* gene cluster from *M. extorquens* AM1 abounds with hairpin structures (4). A conserved hairpin structure has been found between *mauA* and *mauC* in the *mau* gene clusters from the three methylotrophic bacteria studied so far. The *mau* cluster from *M. methylotrophus* W3A1 does not have as many hairpin structures, and it does not contain a hairpin structure downstream of *mauA*.

A strong promoter was identified upstream of the *mau* gene cluster, although the sequences involved are not yet known. Promoters transcribing *mau* genes in diverse methylotrophs are thought to be related, since we have shown that the *mau* gene clusters from *M. methylotrophus* W3A1 and *P. denitrificans* are expressed in *M. extorquens* AM1 (4), presumably from their native promoters. Comparison of nucleotide sequences upstream of the *mauF* genes from *M. extorquens* AM1 and *M. methylotrophus* W3A1 identified two conserved regions (Fig. 5). Further work will be necessary to assess the role of these sequences in transcription of *mau* genes.

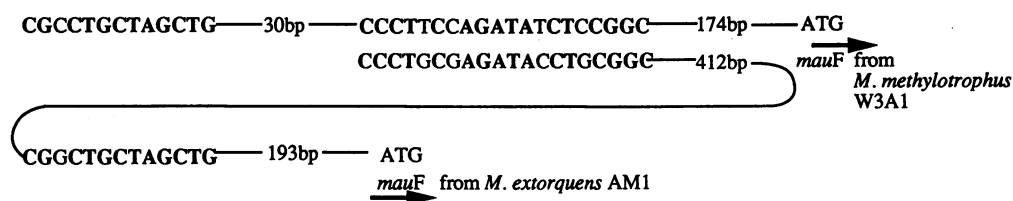


FIG. 5. Alignment of the conserved sequences from the *mau* cluster promoter regions from *M. methylotrophus* W3A1 (upper line) and *M. extorquens* AM1 (lower line). Identical bases are in boldface.

The plasmid generated for these promoter studies, pAYC229, has features that suggest that it may be useful as an expression vector. Four unique sites for *Hind*III, *Cla*I, *Eco*RI, and *Hpa*I between *P_{mau}* and *aphC* and two for *Sac*I and *Sac*II inside *aphC* could be used for cloning DNA fragments with genes to be expressed. Studies are currently under way to determine the utility of pAYC229 as an expression vector for *M. methylotrophus* W3A1.

Substantial similarity exists between *Mau* polypeptides from *M. extorquens* AM1, *P. denitrificans*, *T. versutus*, *Methylobacillus flagellatum* KT, and *M. methylotrophus* W3A1 (17). Identities between polypeptides from *M. methylotrophus* W3A1 and *M. extorquens* AM1 are 49% for *MauF*, 45% for *MauB*, 39% for *MauE*, 52% for *MauD*, 63% for *MauA*, 51% for *MauG*, 31% for *MauL*, and 55% for *MauF*.

Several key features of the *Mau* polypeptides are conserved in these phylogenetically distinct strains. *MauFs* and *MauEs* are predicted to be transmembrane polypeptides with four helices in all cases; *MauGs* are predicted to contain two heme *c* binding sites; *MauMs* are predicted to contain four iron-sulfur clusters; *MauAs* have unusual leader sequences; *MauBs*, *MauMs*, and *MauGs* are predicted to have normal leader sequences, and the leader sequences of *MauD* are devoid of positively charged amino acids. Two exceptions exist: the *MauD* polypeptide from *M. extorquens* AM1 is predicted to be a lipoprotein, whereas *MauD* from *M. methylotrophus* W3A1 is predicted to be a periplasmic polypeptide. Instead, *MauL* is predicted to be a lipoprotein in *M. methylotrophus* W3A1, whereas its counterpart from *M. extorquens* AM1 is predicted to be a periplasmic polypeptide.

The *mau* system was shown here to be the only detected system of methylamine oxidation in *M. methylotrophus* W3A1. Two bacteria studied previously (*M. extorquens* AM1 [4] and *M. flagellatum* KT [11]) have alternative amine oxidation systems which allow them to grow on methylamine as a source of nitrogen in cells in which the MADH system is impaired. *N*-Methylglutamate dehydrogenase activity was found in cell extracts of *M. extorquens* AM1 grown with methylamine as a source of nitrogen (4), but the alternative system in *M. flagellatum* KT has not been identified yet. The *mauA* mutant of *M. methylotrophus* W3A1 was still able to grow on trimethylamine but not on di- or monomethylamine. This result is expected, based on the biochemistry of amine oxidation in *M. methylotrophus* AS1 (2). In this strain, trimethylamine is oxidized via trimethylamine dehydrogenase, which donates electrons to the electron transfer chain and hence is connected to energy metabolism. Dimethylamine is oxidized via dimethylamine monooxygenase, which does not generate energy and therefore is unable to sustain methylotrophic growth.

Key questions about the organization of the *mau* genes in *M. methylotrophus* W3A1 are whether MADH from this bacterium uses cytochrome *c* as an electron acceptor, as has been suggested (3), and if so, where the gene for this cytochrome is located with respect to the *mau* gene cluster. Preliminary data indicate the presence of the gene for a putative monoheme *c*-type cytochrome (*mauO* [17]) approximately 1 kb downstream of *mauM*, which is a candidate as an electron acceptor for MADH.

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